ally related metabolite from combination with its enzyme(9) and thus preventing the formation of an enzyme substrate complex, it is probable that the amethopterin retained for such long periods of time is incorporated up to a relatively constant concentration in enzymes ordinarily associated with folic acid and CF, and any excess is rapidly excreted.

Summary. 1. Amethopterin given parenterally in various doses to normal mice resulted in the retention of a more or less constant amount of amethopterin in the liver for a period of at least 3 weeks. A decrease in the kidney concentration of amethopterin was noted. 2. CF at doses ranging from 0.5 to 30 mg/kg intraperitoneally failed to have any detectable effect 24 hours later on the concentration of amethopterin in the liver and kidneys, but caused a significant rise in the serum level of amethopterin. 3. The inhibitory substance present in the mouse liver 24 hours to 14 days after the administration of amethopterin has the same Rf value as amethopterin in 3 different solvent systems, and is therefore considered to be amethopterin.

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A Colorimetric Method for Hyaluronic Acid Estimation.* (20363)

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Several methods are available for the quantitative measurement of hyaluronic acid. The material can be precipitated, purified, dried and weighed. The viscosity of its aqueous solutions can be measured, although this property varies, depending upon the amount of depolymerization that occurs during isolation and purification(1). The observation that hyaluronic acid forms an insoluble complex with protein at or near pH 4.0 led to the development of the turbidimetric test for hyaluronic acid(2). Although this technique has been widely employed and is currently used (with or without modification) for the measurement of hyaluronidase activity(3-6), the necessity for measuring the optical density of a suspension in which turbidity is increasing with time is a limitation. Under test conditions ordinarily employed turbidity formation proceeds rapidly at first and then at a decreasing rate for 10 to 20 minutes. The time elapsing between the addition of the reagents and the measurement of optical density must therefore be carefully controlled. Furthermore, when large amounts of polysaccharide are present flocculation may occur. The optical density then increases at first

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but soon decreases as the floccules begin to settle to the bottom of the tube. Greif(6) has introduced a colorimetric method for hyaluronic acid which overcomes some of the difficulties. In his procedure, outdated blood bank plasma proteins are coupled to the indicator dye, bromsulfalein. When precipitation with polysaccharide occurs the dye is carried down in the protein-polysaccharide complex. After centrifugation the complex is dissolved in a standard volume of weak alkali. This releases the dye which then imparts to the alkaline solution a stable color that is proportional to polysaccharide concentration over a range of 0.1 to 0.2 mg.

The present report describes a simple colorimetric procedure for the determination of hyaluronic acid based on the co-precipitation of hyaluronic acid and hemoglobin. It is sensitive, accurate, and employs readily reproducible reagents.

Methods. Reagents. (a) 2M sodium acetate-glacial acetic acid buffer, pH 3.8. (b) Hemoglobin solution. This is prepared as follows: twenty-five ml of fresh sheep blood is centrifuged and the sedimented cells are washed three times with three volumes of 0.85% saline. The packed cells from the final centrifugation are suspended and laked in 50 ml of distilled water. After the addition of 2 ml of 25% aqueous sodium chloride, the erythrocyte stromata are removed by centrifugation at 8000 rpm for 30 minutes. The clear supernatant solution is then diluted with 100 ml of distilled water containing sufficient merthiolate to give a final concentration of 0.01%. Solutions thus prepared, if stored at 5°C, will give closely reproducible values in determination of hyaluronic acid for at least 3 weeks. *Substrate.* Streptococcal hyaluronic acid was prepared by the method of Harris and Harris(7).

Procedure. The test system consists of the following: 2 ml hemoglobin solution in distilled water; 0.5 ml sample; 0.5 ml distilled water; 2 ml 2M acetate, pH 3.8, listed in the order of addition of the solutions. The tubes (15 cm long x 11 mm O.D.) are allowed to stand in a refrigerated water bath at 5 to 10°C for one hour while precipitation is completed. The color of the mixtures changes gradually from red to brown as the hemoglobin is oxidized to methemoglobin. After centrifugation at 5-10°C for 15 minutes at 2700 rpm on a horizontal head, the supernates are poured off and the tubes drained 10 minutes in an inverted position. Excess fluid is removed from the rims with absorbent tissue. Five ml of distilled water and 2 drops of approximately 6 N NaOH are added and after the precipitates are redissolved with the aid of a glass stirring rod, approximately 0.02 g of crystalline sodium cyanide is added to each tube. The red color which develops has light absorption peaks at 420 and 545 μ. This suggests a similarity to or identity with ferriheme cyanide(8). Optical density is estimated on a Lumetron photoelectric colorimeter using a 550 μ filter having a 30 μ band width. Intensity of the color is maximal after 10 minutes and remains unchanged for at least one hour.

TABLE I. Hyaluronic Acid Recovery Tests.

Added	Found	Recovered	Max deviation
	mg		
.110	.135	.110	± .002
.110	.135	.110	
.110	.134	.109	
.110	.135	.110	
.440	.454	.429	± .011
.440	.463	.438	
.440	.454	.429	
.440	.463	.438	

Above amounts of purified hyaluronic acid were added to 0.5 ml aliquots of a culture supernate containing 0.05 mg hyaluronic acid/ml.

Preliminary trials to determine the optimal acidity for precipitation indicated that any pH between 3.5 and 4.2 would be equally suitable. The high molarity of the acetate did not interfere with precipitation and served to control effectively any changes in hydrogen ion concentration in the reaction system caused by the introduction of samples of different pH. Alkali and cyanide do not have to be measured with a high degree of accuracy. Considerable variation in the concentrations of these materials can occur without an apparent effect on the intensity of color developed with a given amount of hyaluronic acid. The results of recovery tests to determine the accuracy of the method are included in Table I.

For precise work a new standard curve

should be prepared at biweekly intervals and a standard solution of hyaluronic acid should be included in each determination as a reagent control.

Discussion. The procedure for determining hyaluronic acid described above has been found to offer a number of advantages not found in previously described methods. The reagents employed are easily prepared and readily reproducible. The hemoglobin solution can be standardized colorimetrically. The error is small, and a large number of tests can be carried out without the necessity for careful timing. Furthermore, when it is necessary to measure hyaluronic acid in broth culture supernates, the color of the medium does not interfere. A large series of heart infusion broth media, modified in various ways by the addition of sugars, buffers and growth factors, gave essentially identical blank readings whereas considerable variation would have been found if a turbimetric system had been employed.

A possible variable of the bromsulfalein method not present in the hemoglobin technic is the molar ratio of chromogenic substance (bromsulfalein) to protein. For good reproducibility this relationship must be constant. The molar ratio of heme to globin in native hemoglobin is a constant and thus fulfills this requirement. The accuracy and sensitivity of the method can readily be deduced from Fig. 1 which presents a standard curve with streptococcal capsular material. Color development follows Beer's Law closely over a range of 0.1 to 0.5 mg of polysaccharide. This gives a greater range of maximal accuracy than that found in either the turbidimetric or bromsulfalein procedures. Day to day reproducibility is improved by carrying out precipitation and centrifugation at a standard temperature.

Summary. A new colorimetric method for the determination of hyaluronic acid is described, based upon precipitation of acid polysaccharide with hemoglobin at pH 3.8 and conversion of the prosthetic group of the precipitated protein to ferriheme cyanide. The color of this derivative is determined photoelectrically and the value for hyaluronic acid obtained by reference to a standard curve prepared with known material.

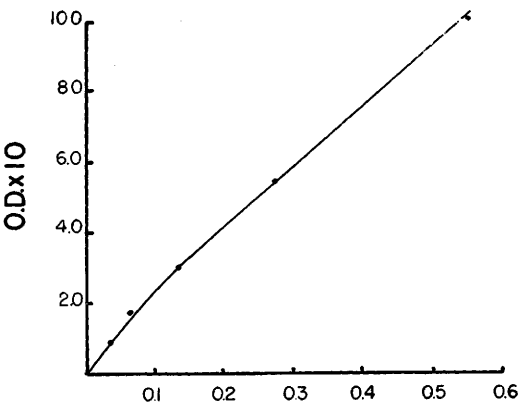

FIG. 1.

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